

¹Improved Synthesis of Sodium D-Glucuronate-6-C¹⁴ and of D-Glucose-6-C¹⁴

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A process is reported for the synthesis of barium 1,2-*O*-isopropylidene-D-glucofururonate-6-C¹⁴ in 55 percent yield from 1,2-*O*-isopropylidene-D-xylo-dialdopentofuranose and sodium cyanide-C¹⁴. The salt was converted to sodium D-glucuronate-6-C¹⁴ in 92 percent radiochemical yield and to D-glucose-6-C¹⁴ in 84 percent yield. The over-all radiochemical yield of D-glucose-6-C¹⁴ based on the sodium cyanide-C¹⁴ used was 45 percent in comparison with prior yields of approximately 15 percent.

1. Introduction

C¹⁴-labeled glucuronic acid and D-glucose-6-C¹⁴ are in demand for biological and medical research but have not been generally available for lack of efficient methods of synthesis. Most of the material heretofore available was made by a procedure originated by Sowden [1].² The procedure begins with 1,2-*O*-isopropylidene-D-glucose and includes the intermediate production of a solution containing 1,2-*O*-isopropylidene-D-xylo-dialdopentofuranose, reaction of this solution with sodium cyanide-C¹⁴, separation of 1,2-*O*-isopropylidene-D-glucofururonic acid, lactonization of the acid, and reduction of the lactone with either sodium borohydride or lithium aluminum hydride [2].

The method was modified by Shafizadeh and Wolfrom [3]. They avoided isolation of 1,2-*O*-isopropylidene-D-glucofururonic acid by lactonizing the acid mixture and separating the epimeric lactones by chromatography on clay. By use of this technique and a bicarbonate buffer in the cyanohydrin reaction they obtained crystalline 1,2-*O*-isopropylidene-D-glucofururano- γ -lactone in 15.5 percent yield and 1,2-*O*-isopropylidene-D-idurono- γ -lactone in 16 percent yield, based on the amount of cyanide used. The modification is a marked improvement over the original Sowden method which gave a 12.5 percent yield of only the glucuronic epimer, but it is scarcely suitable for the commercial production of D-glucose-6-C¹⁴. It is therefore of interest to report a procedure developed at the National Bureau of Standards as part of a general program on the production of position-labeled sugars [4]. The procedure has been used for the preparation of D-glucose-6-C¹⁴ which has been supplied to numerous research groups in this country and abroad.

2. Discussion of the Process

Until recently the starting material for the synthesis, 1,2-*O*-isopropylidene-D-xylo-dialdopentofuran-

ose, was known only as a sirup. It was first prepared by Iwadare [5] from 1,2-*O*-isopropylidene-D-glucofuranose by oxidation with lead tetraacetate. Later, oxidation with sodium metaperiodate was employed [6] and the sirupy product was used for the synthesis of 6-nitro-6-deoxyhexoses [7] and D-xylose-1-C¹⁴ [6] as well as for D-glucose-6-C¹⁴. The sirupy product is not satisfactory for the synthesis of labeled sugars because it contains formaldehyde derived from the glycol oxidation. The formaldehyde may be combined with the sugar; in any case, it adheres tenaciously to the product. In the present investigation the last trace of formaldehyde was removed by crystallization of the product from water.

Crystalline 1,2-*O*-isopropylidene-D-xylo-dialdopentofuranose has been extensively studied and used at the National Bureau of Standards since 1952. The product that crystallizes from water contains 1 mole of water for 4 moles of the aldehyde. The molecular weight determined from freezing point depressions indicates that the product is a dimer. By dissolution of the hydrate in benzene and subsequent crystallization, an anhydrous product was obtained. This substance appears to be the same as a crystalline product recently reported by Brocca and Dansi [8]. Both the hydrate and the anhydrous product are suitable raw materials for the present process. Use of a crystalline compound rather than a crude sirup for starting the process eliminates loss of labeled cyanide by reaction with impurities and greatly simplifies the synthesis.

In prior work on the labeled-sugar program it was found that the proportions of the epimers formed in the cyanohydrin reaction can be altered by use of suitable buffers [9]. To ascertain conditions for the optimum yield of glucuronic acid, a number of cyanohydrin reactions were conducted with the xylose derivative, in which several buffers and C¹⁴-labeled cyanide were used. The conditions employed in these reactions (table 1) were developed in experiments with nonradioactive materials in which the reaction times for complete addition of cyanide and the conditions for hydrolysis of the cyanohydrins were studied. In the work with labeled cyanide the yield of glucuronic acid was determined by an isotope dilution technique. The

¹ Part of a project on the development of methods for the synthesis of radioactive carbohydrates, sponsored by the Atomic Energy Commission. The paper is based on work described in an AEC report.

² Figures in brackets indicate the literature references at the end of this paper.

TABLE 1. Yields of 1,2-*O*-isopropylidene-*D*-glucuronate salts from cyanohydrin reactions as determined by isotope dilution analysis

Part 1. Composition of the cyanohydrin reaction mixtures					
Reaction mixture	1,2- <i>O</i> -Isopropylidene- <i>D</i> -xylo-dialdo-pentofuranose	Sodium cyanide —C ¹⁴	Sodium hydroxide	Other reagents	Water
I-----	<i>mM</i> 0.110	<i>mM</i> 0.089	<i>mM</i> 0.30	<i>mM</i> 0.37 NaHCO ₃	<i>ml</i> 3
II-----	.077	.060	.20	.54 NaHSO ₃	3
III-----	.077	.060	.20	.54 HOAc	3
IV-----	.175	.160	.60	1.4 HOAc	5

Part 2. Conditions and results								
Reaction mixture	Conditions for addition reaction		Conditions for hydrolysis			Radioactivity		Yield of glucuronate
	Temp.	Time	Time at 70° C	Na ₂ CO ₃	Time at 100° C	NaC ¹⁴ N	Glucuronate-6-C ¹⁴	
I-----	°C 7 to 9	Days 5	Hours None	<i>mM</i> None	Hours 3	<i>μ</i> / <i>mM</i> 700	<i>μ</i> / <i>mg</i> 0.160 ^b	Percent 27
II-----	25 to 30	3	2	0.3	3	700	.152 ^b	38
III-----	25 to 30	14	2	.3	3	700	.173 ^c	44
IV-----	25 to 30	25	2 ^a	.7	2	700	.422 ^c	58

^a 0.15 mM of Na₂SO₃ was added before heating was begun.

^b Lithium 1,2-*O*-isopropylidene-*D*-glucuronate-6-C¹⁴ obtained by use of 100 mg of carrier.

^c Barium 1,2-*O*-isopropylidene-*D*-glucuronate-6-C¹⁴ obtained by use of 125 mg of carrier.

results revealed that in the slightly acid solution of mixture IV the reaction is slow but the yield of glucuronic epimer is high, and that with an increase in alkalinity the yield decreases.

Under conditions ordinarily used for hydrolyzing nitriles in the cyanohydrin synthesis, the reaction mixture from the 5-*aldo*-xylose derivative turns dark brown and there is much decomposition. To avoid this complication it was found advantageous to start the hydrolysis at a relatively low temperature (70° C), and at a relatively low alkalinity. After partial hydrolysis, the alkalinity was increased by the addition of sodium carbonate and the temperature was raised to 100° C. It was also found that reaction mixtures containing sodium sulfite gave lighter colored solutions in the hydrolysis step than others. In the procedure finally developed, sodium sulfite is added prior to heating at 70° C and sodium carbonate prior to heating at 100° C. The course of the hydrolysis was followed by titration of the ammonia liberated, or qualitatively by use of moist pH test paper.

To provide a means for separating the epimeric acids formed in the hydrolyzate, the salts of 1,2-*O*-isopropylidene-*D*-glucuronic acid were studied. The previously known barium salt [10] and a new lithium salt were found to crystallize well and to be suitable for isolating 1,2-*O*-isopropylidene-*D*-glucuronic acid from the reaction mixture. The barium salt seems the more useful, but both salts gave clear-cut separations since the corresponding salts of iduronic acid do not crystallize.

In the procedure recommended for the production of labeled glucuronic acid, the cold hydrolyzate is

passed through a column containing a cation exchange resin, and the effluent is neutralized immediately with barium hydroxide. An insoluble residue is separated by filtration and the aqueous filtrate is concentrated to crystallization of barium 1,2-*O*-isopropylidene-*D*-glucuronate. Separation of a pure crystalline salt of 1,2-*O*-isopropylidene-*D*-glucuronic acid makes isolation of crystalline 1,2-*O*-isopropylidene-*D*-glucuronic acid unnecessary, thus eliminating loss of product that would be caused by partial hydrolysis of the isopropylidene groups.

For the production of *D*-glucose-6-C¹⁴ the crystalline salt is converted to the acid by use of an ion-exchange resin. The solution containing the acid is lyophilized and the dry residue is lactonized by heating with toluene according to the method of Sowden [1]. Under these conditions there is little loss due to hydrolysis of the isopropylidene group. The lactonized isopropylidene derivative is reduced without isolation of the pure lactone with either sodium borohydride or with lithium aluminum hydride by the method of Roseman [2]. The isopropylidene-*D*-glucose-6-C¹⁴ thus obtained is converted to *D*-glucose-6-C¹⁴ by hydrolysis and the sugar is separated by crystallization in the usual manner.

For the production of glucuronates, or *D*-glucuronic, the salt of 1,2-*O*-isopropylidene-*D*-glucuronic-6-C¹⁴ acid is heated with a sulfonic acid ion-exchange resin. This converts the salt to the acid and hydrolyzes the isopropylidene group. Sodium, potassium, and other salts of glucuronic acid are then obtained by neutralization of the acid with the corresponding base, followed by crystallization. The lactone (*D*-glucurone-6-C¹⁴) is obtained by lyophilization of the aqueous acid, dissolution of the residue in Methyl Cellosolve (ethylene glycol monomethyl ether), and crystallization.

3. Experimental

3.1. 1,2-*O*-Isopropylidene-*D*-xylo-dialdo-pentofuranose

A stirred solution of 50 g of sodium metaperiodate and 400 ml of water in a 1-liter flask, surrounded by an ice bath, was treated during 30 min with 50 g of 1,2-*O*-isopropylidene-*D*-glucofuranose, added in small portions. After stirring an additional 20 min, the excess periodate was decomposed by the addition of ethylene glycol. The solution was concentrated by freeze drying and the residue was extracted with four 100-ml volumes of chloroform. The extracts were combined, clarified by filtration through carbon, and concentrated under reduced pressure to a heavy sirup. By dissolution of the sirup in 100 ml of water and reconcentration under vacuum, the chloroform was removed. The concentrate was dissolved in 50 ml of water, filtered, and stored in the refrigerator for several weeks. The crystals that formed were separated, washed with water, and then recrystallized from water to give 29.9 g of 1,2-*O*-isopropylidene-*D*-xylo-dialdo-pentofuranose hydrate. Additional hydrate crystallized after lyophilization of the mother liquors and dissolution of the residue in an equal weight of water.

Anal. Calculated for $C_8H_{12}O_5 \cdot \frac{1}{4}H_2O$: C, 49.9; H, 6.5.
Found: C, 50.1; H, 6.6.

The hydrate, which crystallizes from water as large prisms, had a molecular weight of 262 in formamide and of 253 in acetic acid as determined by the Beckman freezing-point method; these values correspond with the calculated value 257 for a dimer with one-half molecule of water of hydration. The product melted at 180° to 184° C when heated slowly from room temperature to the melting point. Immersion of a sample in a capillary tube into a bath whose temperature exceeds 145° C resulted in rapid melting with evolution of gas. The melt solidified and remelted below 184° C. The hydrate gave $[\alpha]_D^{20}$ of -25.6° at equilibrium in water.

By dissolving the hydrate in boiling benzene and cooling, needlelike crystals of anhydrous 1,2-*O*-isopropylidene-*D*-xylo-dialdopentofuranose were obtained. The crystals were separated, washed with a mixture of benzene and petroleum ether, and dried.

Anal. Calculated for $C_8H_{12}O_5$: C, 51.1; H, 6.4.
Found: C, 51.1; H, 6.4.

The anhydrous compound melted at 182° to 184° C, and in water solution at equilibrium $[\alpha]_D^{20} = -25.9^\circ$. The substance had a molecular weight of 390 in acetic acid as determined by the Beckman freezing-point method. The calculated value for the dimer is 376. Brocca and Dansi [8] report a similar product with a melting point of 175° to 177° C and $[\alpha]_D^{30}$ of -26.6° at equilibrium.

3.2. Lithium 1,2-*O*-Isopropylidene-*D*-glucofuranate

To 5 ml of a stirred cold aqueous solution containing 1.08 g of 1,2-*O*-isopropylidene-*D*-glucurone was added 0.21 g of $LiOH \cdot H_2O$. The solution became yellow and in a few moments crystals of the lithium salt appeared. After stirring for 10 min, 20 ml of ethanol was added dropwise during the next half hour. The slightly yellow crystals were separated, washed with 80 percent ethanol, and dried. They weighed 0.93 g. The product was dissolved in water, the solution was filtered through a bed of decolorizing carbon, and crystallization was induced by the gradual addition of ethanol. The crystals obtained were short colorless needles which did not melt below 240° C. $[\alpha]_D^{20} = -3.8^\circ$ (c, 3; H_2O).

Anal. Calculated for $LiC_9H_{13}O_7$: Li, 2.9; C, 45.0; H, 5.5;
Found: Li, 2.9; C, 45.2; H, 5.6.

3.3. Yields of 1,2-*O*-Isopropylidene-*D*-glucuronate Salts from 1,2-*O*-Isopropylidene-*D*-xylo-dialdopentofuranose and C^{14} -labeled Cyanide

The compositions of the reaction mixtures are given in part 1 of table 1, and a summary of the conditions employed and the data from which the yields were calculated is given in part 2. The re-

actants of each mixture were combined as solutions and stored in stoppered containers at the indicated temperatures. At the end of the time allowed for completion of the addition reaction, mixtures II and III were warmed to 70° C, and mixture IV, after addition of sodium sulfite, was also heated to 70° C. Mixtures II, III, and IV were then treated with sodium carbonate and all four reaction mixtures heated in a boiling water bath to complete the hydrolysis of the cyanohydrins. Nonradioactive lithium 1,2-*O*-isopropylidene-*D*-glucuronate was added to I, II, and III as carrier; the corresponding barium salt to IV. After cooling in ice water each solution was passed through a column containing 5 ml of Amberlite 1R-120H resin.³ The effluents of I, II, and III were neutralized with lithium hydroxide. Barium hydroxide was used for IV and the insoluble matter removed. The neutralized effluents were concentrated under vacuum until the onset of crystallization, warmed to redissolve any material that had separated, and then passed through a filter coated with decolorizing carbon. The filtrates were concentrated under an air stream, seeded with the appropriate isopropylidene-glucuronate salt, and, as the crystallizations proceeded, treated with small amounts of methanol. The products were washed with aqueous methanol and then recrystallized from water and methanol. The specific activities of the isolated salts are given in table 1 together with the calculated yields for each reaction mixture.

3.4. Barium 1,2-*O*-Isopropylidene-*D*-glucuronate- C^{14}

A solution of 5.0 mM of sodium cyanide containing 12.5 mc of C^{14} and 5 mM of sodium hydroxide in 25 ml of water was frozen on the side of a 200-ml flask. Twenty-five ml of an aqueous solution containing 1.11 g. of 1,2-*O*-isopropylidene-*D*-xylo-dialdopentofuranose was introduced and frozen in the flask. Finally 20 ml of 1.0 *N* acetic acid was added and the flask was securely stoppered and swirled until its contents were completely melted. After 25 days at room temperature the flask was opened, 0.252 g of Na_2SO_3 was added, and the slightly yellow solution was heated at 70° C for 2 hr. After the addition of 1.06 g of Na_2CO_3 the solution was refluxed for 2 hr, next cooled in an ice bath, and then passed through a column containing 50 ml of cation exchange resin (Amberlite 1R-120H) at ice temperature. The effluent, including washings, was delivered into a flask surrounded by ice, containing 7.88 g of $Ba(OH)_2 \cdot 8H_2O$. A stream of gaseous carbon dioxide was next passed into the effluent until the solution was neutral and the insoluble material was separated by filtration.

Concentration of the filtrate under reduced pressure to about 60 ml resulted in the separation of an additional amount of precipitate. Refiltration yielded a solution which contained according to a radioassay 11.76 mc of C^{14} . A few seed crystals of

³ Amberlite 1R-120-H, Resinous Products Division of Rohm & Haas Co., Philadelphia, Pa.

barium 1,2-*O*-isopropylidene- β -glucuronate [10] were added and the solution was then concentrated under a gentle stream of air. Large colorless crystals of labeled barium salt formed during the evaporation. They were separated from the mother liquor and washed several times with water and 50 percent methanol. Dissolution of the crystalline product in water, removal of a small amount of insoluble residue, and recrystallization yielded 0.690 g of barium 1,2-*O*-isopropylidene- β -glucuronate-6- C^{14} monohydrate containing 5.6 mc of radioactivity. Three 500-mg portions of nonradioactive barium salt used as carrier resulted in the isolation of 0.86 mc, 0.22 mc, and 0.07 mc of additional salt. The radiochemical yield (6.75 mc) amounted to 54 percent based on the $NaC^{14}N$ used. The mother liquor (5.0 mc) was set aside for separation of 1,2-*O*-isopropylidene- β -iduronate-6- C^{14} epimer.

3.5. Preparation of 1,2-*O*-Isopropylidene- β -glucuronolactone-6- C^{14} From Barium 1,2-*O*-Isopropylidene- β -glucuronate-6- C^{14}

Seventy ml of an ice-cold aqueous solution of 1.184 g of barium 1,2-*O*-isopropylidene- β -glucuronate-6- C^{14} monohydrate containing 6.5 mc of C^{14} was passed through a column containing 9 ml of ice-cold Amberlite 1R-120H. The effluent and washings were frozen and lyophilized. A volume of 100 ml of toluene was added to the sirup which remained after the lyophilization. The mixture was heated under a reflux condenser for 4 hr. Removal of the toluene by evaporation under a gentle stream of air left large crystals of 1,2-*O*-isopropylidene- β -glucurone-6- C^{14} mixed with some brown residue.

3.6. Preparation of β -Glucose-6- C^{14} From 1,2-*O*-Isopropylidene- β -glucuronolactone-6- C^{14}

The crude 1,2-*O*-isopropylidene- β -glucurone-6- C^{14} described in the preceding paragraph was dissolved in approximately 70 ml of anhydrous diethyl ether and then added at a dropwise rate from a pressure-equalized dropping funnel to a flask containing 13.2 mM of lithium aluminum hydride in 30 ml of diethyl ether. The solution was stirred continuously with a magnetic stirrer. The residual labeled material was rinsed into the reduction flask with ether and the reaction mixture was refluxed for 30 min. Then 25 ml of anhydrous ethanol was added dropwise to the stirred reaction mixture, followed by 6 ml of glacial acetic acid and 130 ml of water. The supernatant ether was volatilized by a current of air. The resulting aqueous solution was passed into a column containing 100 ml of cation exchange resin (Amberlite 1R-120H). The effluent including wash liquor was concentrated at reduced pressure, 10 mM of aqueous sulfuric acid added, and the volume adjusted to 200 ml. The solution was heated for 2 hr in a boiling water bath, and, after cooling to room temperature was passed into a column containing, 40 ml of anion exchange resin.⁴ The radioactive solution was washed from the resin, concentrated to about 50 ml

filtered through a bed of decolorizing carbon and lyophilized. The residue was dissolved in the minimum quantity of methanol and the solution was filtered. Crystalline β -glucose-6- C^{14} was obtained after addition of 2-propanol to the point of incipient turbidity. Recrystallization yielded 485 mg of β -glucose-6- C^{14} with a specific radioactivity of 9.2 μ c/mg. By the use of 2.0 g of carrier, an additional 993 μ c of the labeled glucose was obtained. The chemical yield from the barium salt to the sugar was 69 percent; the radiochemical yield was 84 percent. The over-all radiochemical yield based on the sodium cyanide- C^{14} used was 45 percent.

3.7. Preparation of Sodium β -Glucuronate-6- C^{14} Monohydrate From Barium 1,2-*O*-Isopropylidene- β -Glucuronate-6- C^{14}

In a 100-ml flask equipped with a magnetic stirrer, an electric heater, and a reflux condenser, 896 mg of barium 1,2-*O*-isopropylidene- β -glucuronate (590 μ c), 10 ml of Amberlite 1R-120H, and 40 ml of water were combined and heated to boiling for 50 min. The mixture was cooled, filtered through a small bed of resin, and washed. The filtrate was neutralized with sodium hydroxide, concentrated under reduced pressure, treated with decolorizing carbon, and then filtered. The concentrated filtrate was diluted with a few drops of ethanol, and seeded with crystals of sodium β -glucuronate monohydrate [11]. The resulting crystals were separated from the mother liquor and washed with aqueous ethanol. Recrystallization from aqueous ethanol yielded 595 mg of the monohydrate with a total radioactivity of 509 μ c. By use of nonradioactive carrier an additional 34 μ c of sodium β -glucuronate-6- C^{14} was obtained from the mother liquor. The radiochemical yield was 92 percent.

3.8. Preparation of β -Glucurone-6- C^{14} From Barium 1,2-*O*-Isopropylidene- β -Glucuronate-6- C^{14}

A solution of 927 mg of barium 1,2-*O*-isopropylidene- β -glucuronate-6- C^{14} (165 μ c) in 50 ml of water, and 10 ml of Amberlite 1R-120H resin, was treated as described for the preparation of sodium β -glucuronate-6- C^{14} . After separation from the resin the solution was concentrated at reduced pressure, passed through a filter coated with decolorizing carbon, and then freeze-dried. The residue was dissolved in Methyl Cellosolve, and the solution was seeded with nonradioactive β -glucurone. Recrystallization gave 438 mg of β -glucurone-6- C^{14} with a total radioactivity of 133.5 μ c. By use of carrier an additional 19.3 μ c of labeled glucurone was obtained. The radiochemical yield was 93 percent.

4. Radioactivity Measurements

Determinations of C^{14} were made upon samples dissolved in formamide or in aqueous mixtures [12]. The radioactivities of the solutions were measured with a proportional beta counter.⁵

⁴ Duolite A-4, Chemical Process Co., Redwood City, Calif.

⁵ Nuclear Measurements Corporation, Indianapolis, Ind., Model PC-1.

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